LIGAND-TARGETED EMULSIONS CARRYING BIOACTIVE AGENTS

Background of the Invention

This invention relates to ligand-targeted emulsions that incorporate biologically active agents on or in their particle surface, and more particularly, to such novel emulsions that are especially useful for the treatment of disease with bioactive agents that have improved risk/benefit profiles when applied specifically to selected cells, tissues or organs.

As used herein, the following terms have the definitions set forth:

Direct conjugation of ligand to the emulsion particle refers to the preparation of a ligand-particle complex before administration wherein the ligand is either adsorbed through ionic, electrostatis, hydrophobic or other noncovalent means to the particle surface (e.g. acylated-antibody), or chemically linked to the surface through covalent bonds to a component of the lipid surface such as a "primer material" (e.g. thio-ether or ester bond), or intrinsically incorporated into the lipid surfactant membrane as a component of the membrane (e.g. a lipid derivatized to a peptiodomimetic agent).

Indirect conjugation refers to the use of avidin biotin where the complex is formed *in vivo* in two or more steps. An example would be giving the biotinylated antibody first, followed by avidin, and followed by the biotinylated emulsion particle. Any other sequential multistep chemical linking system that could be utilized *in vivo* is envisioned to produce the same end result, i.e. the close and specific apposition of the emulsion particle to a targeted cell or tissue surface.

Primer material refers to any constituent or derivatized constituent incorporated into the emulsion lipid surfactant layer that could be chemically utilized to form a covalent bond between the particle and a targeting ligand or a component of the targeting ligand (if it has subunits).

Prolonged association of the emulsion particle with the surface of the targeted cell or tissue is in contradistinction to the transient interaction that an unbound particle, existing free in extracellular body fluids, would achieve. By binding the particle to the cell surface, the continued circulation of the nanoparticle through the body is halted. The affixed particle is able to interact with the target cell surface over an extended period of time. The exact amount of time may be variable, but is meant to exceed that of more transient nontargeted contact between particles and cell surfaces by orders of magnitude.

Surfactant is a term derived from SURFace ACTive AgeNT. A Surfactant is a compound that contains a hydrophilic and a hydrophobic segment. When added to water or solvents, a surfactant reduces the surface tension of the systems for the following purposes emulsifying or dispersing in the present application. Our preferred surfactants are

5   phospholipids and cholesterol but include those lipids that are mentioned in our previous application and the additional detergents specified in our invention disclosure.

Ligand is a molecule that binds to another molecule, used in this application to refer to a small targeting molecule that binds specifically to another molecule on a biological surface separate and distinct from the emulsion particle itself. The reaction does not require

10   nor exclude a molecule that donates or accepts a pair of electrons to form a coordinate covalent bond with a metal atom of a coordination complex.

Emulsion technology is very old and distinct from the more modern liposome technology. This is exemplified by the prolific research and patent literature involving liposomes since the 1963 report by Bangham (Physical structure and behavior of lipids and

15   lipid enzymes., Adv Lipid Res, 1963; 1:65-104). Bangham originally characterized emulsions as "either temporary or permanent dispersions of oils or hydrophobic material in water or vice versa" and liposomes as "... 'myelins' and 'myelinics' ... irrevocably associated with the structures obtained when certain phospholipids are dispersed in water. ... The unit structure is a biomolecular tube of lipids, separated from its adjacent concentric tube by a

20   layer of water." In later years liposomes have been elegantly described as "vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules ... (which) form spontaneously when these lipids are dispersed in aqueous media. ... The liposome membrane forms a bilayer structure which is in principle identical to the lipid portion of natural cell membranes." Liposomes may be prepared by a variety of techniques

25   and have single or multiple membrane layers. They are distinctly different and more complex than emulsions.

Drugs can be incorporated into liposomes within either the internal aqueous phase or within one or more of the lipid bilayer membranes and liposomes can be coupled to ligands of various types. Because of the bilayer nature of a liposome membrane, lipophilic drugs

30   incorporate into both the inner and outer leaflets of the bilayer. Drugs, bound to the inner leaflet layer are unavailable for immediate delivery by contact facilitated delivery as opposed to lipid encapsulated emulsions. For multilamellar liposomes, most of the drug will be internalized within the liposome and not readily available for contact facilitated delivery to a target cell. To extend circulatory half-life, liposomes have been modified with polymerized

lipids or the addition of polyethylene glycol to enhance in vivo survivability. Both modifications protect the particles from lipid exchange with other cells and lipoproteins.

"An emulsion is a heterogeneous system, consisting of at least one immiscible liquid intimately dispersed in another in the form of droplets, whose diameters, in general, exceed

5   0.1 μ. *Such systems possess a minimal stability, which may be accentuated by such additives as surface-active agents, finely-divided solids, etc.*" (Becher P. Emulsion: Theory and Practice, New York, NY; Reinhold Publishing Corporation; 1965) "The phase which is present in the form of finely divided droplets is called the dispersion or internal phase; the phase which forms the matrix in which these droplets are suspended is called the continuous

10   or external phase. ... Surface active or other agents which are added to increase stability ... are known as emulsifiers or emulsifying agents. Stability is also increased by mechanical devices such as simple stirrers, homogenizers or colloid mills."

Liquid perfluorocarbon emulsions are specialized formulations with various medical and oxygen transport applications. They are especially useful medically as contrast media, for

15   various biological imaging modalities such as nuclear magnetic resonance, ultrasound, x-ray, computed tomography, F-magnetic resonance imaging, and position emission tomography, as oxygen transport agents or "artificial bloods," in the treatment of heart attack, stroke, and other vascular obstructions, as adjuvants to coronary angioplasty and in cancer radiation treatment and chemotherapy. The fluorocarbon emulsion can be used to deliver drugs and

20   medicines soluble in or transportable by the emulsion.

Long et al. U.S. patent no. 4,987,154 discloses that fluorocarbon emulsions can deliver therapeutic agents, medicines and drugs throughout the body, tissue and organs by at least two modes: 1) within the fluorocarbon phase or 2) by complexing of the agent, *medicine or drug with the surfactant membrane. Long et al. cite examples of medicines,*

25   drugs and therapeutic agents that can be dissolved in the fluorocarbon including diazepam, cyclosporin, rifampin, clindamycin, isoflurane, halothane and enflurane. Examples of medicines, therapeutic agents and drugs that do not dissolve in fluorocarbon, but can be complexed with, for example, a lecithin membrane include mannitol, tocopherol, streptokinase, dexamethasone, prostaglandin E, interleukin, gentamycin and cefoxitin.

30   Antibiotics may be delivered transcutaneously through the skin when added to a fluorocarbon emulsion. Furthermore, proteins such as thrombolytic agents, hormones or enzymes can be transported and delivered by fluorocarbon emulsions.

Delivery of drugs as described by Long et al. and others depend upon the encapsulated drug being more slowly metabolized and eliminated from the circulation than

free drug. In other cases, the encapsulated particles are sequestered into organs and cells involved with the normal metabolism and clearance of particles and foreign matter from the body, a process referred to as passive targeted delivery. The opportunity to conjugate ligands to perfluorocarbon emulsions for the purpose of contact facilitated delivery of bioactive

5   agents was not envisioned by Long et al.

The we have previously reported a novel ligand-targeted, lipid-encapsulated nongaseous perfluorocarbon emulsion useful for ultrasound, magnetic resonance and nuclear imaging applications (U.S. patent nos. 5,690,907, 5,780,010, 5,958,371 and 5,989,520). The perfluorocarbon emulsion is produced through microfluidization techniques, and is robustly

10   stable to handling, pressure, atmospheric exposure, heat and shear. In the early phases of development, we coupled a pretargeted biotinylated ligand to a biotinylated version of the emulsion nanoparticle through avidin-biotin interactions. Subsequently, we adopted a direct ligand conjugation approach using monoclonal $F_{(ab)}$ fragments to facilitate future clinical implementations.

15   The emulsion nanoparticles have long circulatory half-lives due to their small size and inherent *in vivo* stability without further modification of their outer lipid surfaces with polyethylene glycol or incorporation of polymerized lipids. Surfactant modifications often detract from targeting efficacy in order to extend circulatory persistence. The *in vivo* clearance of the nanoparticles was measured in dogs by quantification of the blood

20   perfluorocarbon content with an estimated half-life of one hour. Preliminary data suggest that this novel agent will persist bound to tissue for hours, and dependent upon location, even days.

Millbrath et al [U.S. patent no. 5,401,634] disclose fluorochemical emulsions comprised of a fluorochemical discontinuous phase and aqueous continuous phase with at

25   least one specific binding species immobilized on the droplets. The emulsions can include a "primer material" to couple specific binding species to the fluorochemical droplets. The emulsions may be used in diagnostic procedures or biochemical reactors where binding of the immobilized specific binding species to its binding partner is desired. The droplets were envisioned to incorporate a species (e.g. dye) that is detectable by spectrophotometric,

30   fluorometric or colormetric means. These inventors were focused upon in vitro applications and were unconcerned with in vivo targeted drug delivery. Moreover, they never conceived of the benefits of contact facilitated drug or gene delivery achieved through ligand-targeted emulsion technology.

Magdassi et al, (published PCT application WO 95/03829) described the production and use of ligand-targeted oil emulsions in which drug is "dissolved, dispersed or solubilized inside the oil droplet, creating a novel drug targeting system". The targeted particles provide an oil encapsulated depot of drug at the target site. Subsequent breakdown of the particles

5   releases drug to the interstium. The agent is diluted in extracellular fluids and can migrate from the interstitium to the target cell. Magdassi et al. do not conceive of the utility of contact facilitated delivery of drug or genes from an outer surfactant layer to target tissues. They do not recognize the importance or advantages of a biocompatible phospholipid surfactant layer amenable to exchanging constituents with the target cell membrane. Contact

10   facilitated drug delivery with ligand-targeted emulsions places all of the drug into a monolayer surrounding the particle, ready to interact with the target surface. Mobility of the phospholipid monolayer over the particle surface allows drug from all regions of the layer to migrate and interact with target cell surfaces. Encapsulating drug within the particle, as described by Magdassi et al. isolates the agent from the particle surface and prevents contact

15   facilitated delivery to the target cell membrane.

Unger et al (U.S. patent no. 5,542,935) have described therapeutic delivery systems for site-specific delivery of bioactive agents using gas-filled perfluorocarbon microspheres. The microspheres contain a temperature activated gaseous precursor that becomes a gas upon activation at a selected temperature. Once the microspheres have been introduced into the

20   patient's body, a therapeutic compound may be targeted to specific tissues through the use of sonic energy, microwave energy, magnetic energy, or hyperthermia, which is directed to the target area and causes the microspheres to rupture and release the therapeutic compound. Perfluorocarbons preferred include perfluoromethane, perfluoroethane, perfluorobutane, perfluoropentane, perfluorohexane; even more preferably perfluoroethane, perfluoropentane,

25   perfluoropropane, and perfluorobutane. Unger et al. notes that the localization of these particles for cavitation can be improved with conjugated ligand and the process of active targeting.

There remains a need for improved bioactive agent delivery systems which provide enhanced efficiency of such bioactive agents to targeted tissues, cells or organs.

30   Summary of the Invention

Among the several objects of the invention may be noted the provision of novel compositions and methods for use in delivering bioactive agents to targeted tissues or cells;

the provision of such compositions which provide enhanced delivery of bioactive agents to targeted tissues or cells; the provision of such methods which provides enhanced intermingling and exchange of lipid components from one lipid surface to the other thereby facilitating the exchange of bioactive agents within or on the bioactive agent/emulsion surface

5   to the target cells or tissues; and the provision of such compositions and methods which may be readily practiced. Other objects will be in part apparent and in part pointed out hereinafter.

Briefly, in one aspect, the present invention is directed to a composition for use in delivering a bioactive agent to targeted tissues or cells comprising:

    (a)   site-specific targeting ligand;

10   (b)   a lipid encapsulated oil in water emulsion; and

    (c)   a bioactive agent in or on the surface of the outer lipid monolayer of said emulsion, said ligand being conjugated directly or indirectly to said emulsion and the composition providing facilitated delivery of the bioactive agent through prolonged association and increased contact of the ligand-bound, lipid encapsulated emulsion particles

15 with the lipid bilayer of said target tissues or cells. The invention is also directed to a method for improved delivery of a bioactive agent to targeted tissues or cells comprising administering the above-noted compositions to said tissues or cells.

Brief Description of the Drawings

Fig. 1 is a graph showing that tissue factor-targeted doxorubicin (DXR) nanoparticles

20 had greater anti-proliferative effects than free doxorubicin and that this differential effect was greater at the lower dosage of doxorubicin;

Fig. 2 is a graph showing the effectiveness of therapeutic paclitaxel (Taxol) nanoparticle emulsions targeted to tissue factor on porcine aortic smooth muscle cells in accordance with the present invention; and

25 Fig. 3 is a graph showing the enhanced effectiveness of therapeutic doxorubicin (DXR) or paclitaxel (Taxol) nanoparticle emulsions targeted to tissue factor on porcine aortic smooth muscle cells.

Description of the Preferred Embodiments

In accordance with the present invention, it has now been found that improved

30 compositions for use in delivering a bioactive agent to targeted tissues or cells may be

formulated by combining (a) a site-specific targeting ligand; (b) a lipid encapsulated oil in water emulsion; and (c) a bioactive agent in or on the surface of the outer lipid monolayer of the emulsion. The present invention thus relates to ligand-targeted emulsions that incorporate biologically active agents on or in the outer monolayer of the lipid-based emulsion particle

5   surface. These novel emulsions are particularly useful for the treatment of disease with biological agents that have improved risk/benefit profiles when applied specifically to selected cells, tissues or organs. Site-directed, lipid encapsulated emulsions provide a unique opportunity to deliver potent bioactive agents, such as chemotherapeutic agents, nucleic acid-based therapy, protein-or peptide therapy and the like, with enhanced efficiency to targeted

10   tissues through a unique form of bioactive agent transfer into target cells, i.e. *contact facilitated delivery*. Contact facilitated delivery of bioactive agents by targeted lipid encapsulated emulsions reflects the prolonged association and increased contact of the ligand-bound, lipid-encapsulated particles with the lipid bilayer of the target cell. Enhanced intermingling and exchange of lipid components from one lipid surface to the other facilitates

15   the exchange of bioactive agents within or on the therapeutic emulsion surface to the target cell. It will be understood that the bioactive agent is in or on the surface of the outer lipid monolayer of the emulsion and is preferably, in accordance with the invention, not carried or deposited in the interior of the emulsion particles. It will also be understood that in accordance with the present invention, the emulsion must be nongaseous, i.e. should be a

20   liquid emulsion.

Prolonged association of the emulsion particles with the surface of the targeted cell or tissue is in contradistinction to the transient interaction that an unbound particle, existing free in extracellular body fluids, would achieve. By binding the particle to the cell surface, the continued circulation of the nanoparticle through the body is halted. The affixed particle is

25   able to interact with the target cell surface over an extended period of time. The exact amount of time may be variable, but is meant to exceed that of more transient nontargeted contact between particles and cell surfaces by orders of magnitude.

Targeted therapeutic emulsion may be delivered and concentrated at desired sites in vivo using active-targeting techniques. Active targeting refers to ligand-directed, site-specific

30   accumulation of agents to cells, tissues or organs by localization and binding to molecular epitopes, ie, receptors, lipids, peptides, cell adhesion molecules, polysaccharides, biopolymers, and the like, presented on the surface membranes of cells or within the extracellular matrix. A wide variety of ligands, including but not limited to antibodies, antibody fragments, peptides, small molecules, polysaccharides, nucleic acids, aptamers,

peptidomimetics, other mimetics and drugs alone or in combination may be utilized to specifically bind to cellular epitopes and receptors. These ligands may be attached covalently (direct-conjugation) or noncovalently (indirect conjugation) to the acoustic particle surface.

Avidin-biotin interactions are extremely useful, noncovalent targeting systems that
5   have been incorporated into many biological and analytical systems and selected *in vivo* applications. Avidin has a high affinity for biotin (10^{-15}M) facilitating rapid and stable binding under physiological conditions. Targeted systems utilizing this approach are administered in two or three steps, depending on the formulation. Typically, a biotinylated ligand, such as a monoclonal antibody, is administered first and "pretargeted" to the unique
10   molecular epitopes. Next, avidin is administered, which binds to the biotin moiety of the "pretargeted" ligand. Finally, the biotinylated agent is added and binds to the unoccupied biotin-binding sites remaining on the avidin thereby completing the ligand-avidin-emulsion "sandwich". The avidin-biotin approach can avoid accelerated, premature clearance of targeted agents by the MPS system secondary to the presence of surface antibody.
15   Additionally, avidin, with four, independent biotin binding sites provides signal amplification and improves detection sensitivity.

Targeting ligands may be chemically attached to the surface of acoustic or emulsion particles by a variety of methods depending upon the nature of the ligand and composition of the particle surface. A ligand is a molecule that binds to another molecule, and as used in this
20   application refers to a small targeting molecule that binds specifically to another molecule on a biological surface separate and distinct from the emulsion particle itself. The reaction does not require nor exclude a molecule that donates or accepts a pair of electrons to form a coordinate covalent bond with a metal atom of a coordination complex. Conjugations may be performed before or after the emulsion particle is created depending upon the ligand
25   employed. Direct chemical conjugation of ligands to proteinaceous agents often take advantage of numerous amino-groups (e.g. lysine) inherently present within the surface. Alternatively, functionally active chemical groups such as pyridyldithiopropionate, maleimide or aldehyde may be incorporated into the surface as chemical "hooks" for ligand conjugation after the particles are formed. Another common post-processing approach is to
30   activate surface carboxylates with carbodiimide prior to ligand addition. The selected covalent linking strategy is primarily determined by the chemical nature of the ligand. Monoclonal antibodies and other large proteins may denature under harsh processing conditions; whereas, the bioactivity of carbohydrates, short peptides, aptamers, drugs or peptidomimetics often can be preserved. To ensure high ligand binding integrity and

maximize targeted particle avidity flexible polymer spacer arms, e.g. polyethylene glycol, amino acids or simple caproate bridges, can be inserted between an activated surface functional group and the targeting ligand. These extensions can be 10nm or longer and minimize interference of ligand binding by particle surface interactions.

5   *Monoclonal antibody and fragments*: Rapid expansion of the monoclonal antibody industry has prepared the stage for the clinical success of site-targeted agents by providing a plethora of ligands that can be directed against a wide spectrum of pathologic molecular epitopes. Antibodies or their fragments may be from several classes including IgG, IgM, IgA, IgE or IgD. Immunoglobin-γ (IgG) class monoclonal antibodies have been most often

10   conjugated to liposomes, emulsions and other microbubble particles to provide active, site-specific targeting. These proteins are symmetric glycoproteins (MW ca. 150,000 daltons) composed of identical pairs of heavy and light chains. Hypervariable regions at the end of each of two arms provide identical antigen-binding domains. A variably sized branched carbohydrate domain is attached to complement-activating regions, and the hinge area

15   contains particularly accessible interchain disulfide bonds that may be reduced to produce smaller fragments.

Bivalent F(ab')$_2$ and monovalent F(ab) fragments are derived from selective cleavage of the whole antibody by pepsin or papain digestion, respectively. Elimination of the Fc region greatly diminishes the immunogenicity of the molecule, diminishes nonspecific liver

20   uptake secondary to bound carbohydrate, and reduces complement activation and resultant antibody-dependent cellular toxicity. Complement fixation and associated cellular cytotoxicity can be detrimental when the targeted site must be preserved or beneficial when recruitment of host killer cells and target-cell destruction is desired (e.g. anti-tumor agents).

Most monoclonal antibodies are of murine origin and are inherently immunogenic to

25   varying extents in other species. Humanization of murine antibodies through genetic engineering or other combinatorial chemical methods have led to development of chimeric ligands with improved biocompatibility and longer circulatory half-lives. The binding affinity of recombinant antibodies to targeted molecular epitopes can be occasionally improved with selective site-directed mutagenesis of the binding idiotype.

30   *Phage display*: Phage display techniques are now used to produce recombinant human monoclonal antibody fragments against a large range of different antigens without involving antibody-producing animals. In general, cloning creates large genetic libraries of

corresponding DNA (cDNA) chains deducted and synthesized by means of the enzyme
"reverse transcriptase" from total messenger RNA (mRNA) of human B lymphocytes.
Immunoglobulin cDNA chains are amplified by PCR (polymerase chain reaction) and light
and heavy chains specific for a given antigen are introduced into a phagemid vector.

5   Transfection of this phagemid vector into the appropriate bacteria results in the expression of
a scFv immunoglobulin molecule on the surface of the bacteriophage. Bacteriophages
expressing specific immunoglobulin are selected by repeated immunoadsorption/phage
multiplication cycles against desired antigens (e.g., proteins, peptides, nuclear acids, and
sugars). Bacteriophages strictly specific to the target antigen are introduced into an

10   appropriate vector, (e.g., Escherichia coli, yeast, cells) and amplified by fermentation to
produce large amounts of human antibody fragments with structures very similar to natural
antibodies. Although this technology is still in early stages of development, it has already
permitted the production of unique ligands for targeting and therapeutic applications.
(de Bruin et al., Selection of high-affinity phage antibodies from pahage display libraries.

15   Nat Biotechnol. 1999; 17:397-399; Stadler, Antibody production without animals. Dev Biol
Stand. 1999; 101:45-48; Wittrup, Phage on display, Trends Biotechnol. 1999; 17:423-424;
Sche et al., Display cloning: functional identification of natural product receptors using
cDNA-phage display. Chem Biol. 1999; 6:(707-716).

Peptides: Peptides, like antibodies, may have high specificity and epitope affinity for
20   use as vector molecules for targeted contrast agents. These may be small peptides (5 to 10
amino acids) specific for a unique receptor sequences (e.g. such as the RGD epitope of the
platelet GIIbIIIa receptor) (Wright et al., Evaluation of New thrombus-specific ultrasound
contrast agents. Acad Radiol. 1998; 5 (Supp 1): S240-S242) or larger, biologically active
hormones such as cholecystokinin (Reimer et al., Pancreatic receptors: initial feasibility
25   studies with a targeted contrast agent for MR imaging; Radiology, 1994; 193:527-531).
Smaller peptides potentially have less inherent immunogenicity than nonhumanized murine
antibodies. Peptides or peptide (nonpeptide) analogues of cell adhesion molecules, cytokines,
selectins, cadhedrins, Ig superfamily, integrins and the like may be utilized for targeted
therapeutic delivery.

30   *Asialoglycoproteins and polysaccharides*: Asialoglycoproteins (ASG) have been
used for liver-specific applications due to their high affinity for ASG receptors located
uniquely on hepatocytes (Reimer et al., Preclinical assessment of hepatocyte-targeted MR

constrast agents in stable human liver cell cultures, J Magn Reson Imaging, 1998; 8:687-698
and Leveille-Webster et al., Use of an asialoglycoprotein receptor-targeted magnetic
resonance agent to study changes in receptor biology during liver regeneration and
endotoxemia in rats., Hepatology 1996; 23:1631-1641). ASG directed agents (primarily MR

5   agents conjugated to iron oxides) have been used to detect primary and secondary hepatic
tumors as well as benign, diffuse liver disease such as hepatitis (Reimer et al., Dynamic
signal intensity changes in liver with superparamagnetic MR constrast agents., J Magn Reson
Imaging, 1992; 2:177-181 and Reimer et al., Receptor-directed contrast agents for MR
imaging preclinical evaluation with affinity assays., Radiology, 1992; 182:565-569). The

10   ASG receptor is highly abundant on hepatocytes, approximately 500,000 per cell, rapidly
internalizes and is subsequently recycled to the cell surface. Polysaccharides such as
arabinogalactan may also be utilized to localize agents to hepatic targets. Arabinogalactan
has multiple terminal arabinose groups that display high affinity for ASG hepatic receptors
(Leveille-Webster et al., supra and Small et al., Enhancement effects of a hepatocyte receptor-

15   specific MR contrast agent in an animal model., J Magn Reson Imaging, 1994; 4:325-330).

Aptamers: Aptamers are high affinity, high specificity RNA or DNA-based ligands
produced by *in vitro* selection experiments (SELEX: systematic evolution of ligands by
exponential enrichment) (Small et al., supra).

Aptamers are generated from random sequences of 20 to 30 nucleotides, selectively

20   screened by absorption to molecular antigens or cells, and enriched to purify specific high
affinity binding ligands. To enhance *in vivo* stability and utility, aptamers are generally
chemically modified to impair nuclease digestion and to facilitate conjugation with drugs,
labels or particles. Other, simpler chemical bridges often substitute nucleic acids not
specifically involved in the ligand interaction. In solution aptamers are unstructured but can

25   fold and enwrap target epitopes providing specific recognition. The unique folding of the
nucleic acids around the epitope affords discriminatory intermolecular contacts through
hydrogen bonding, electrostatic interaction, stacking, and shape complementarity. In
comparison with protein-based ligands, aptamers are stable, are more conducive to heat
sterilization, and have lower immunogenicity. Aptamers are currently used to target a

30   number of clinically relevant pathologies including angiogenesis, activated platelets, and
solid tumors and their use is increasing. The clinical effectiveness of aptamers as targeting
ligands for therapeutic emulsion particles may be dependent upon the impact of the negative
surface charge imparted by nucleic acid phosphate groups on clearance rates. Previous

research with lipid-based particles suggest that negative zeta potentials markedly decrease liposome circulatory half-life, whereas, neutral or cationic particles have similar, longer systemic persistence.

The oil phase of the oil in water emulsion may be a vegetable oil, medium chain triglycerides (MCT) or any other oil, with increased or decreased polarity and hydrophobicity or fluorochemical liquid. Suitable fluorochemical liquids include straight and branched chain and cyclic perfluorocarbons, straight and branched chain and cyclic perfluoro tertiary amines, straight and branched chain and cyclic perfluoro ethers and thioethers, halofluorocarbons and polymeric perfluoro ethers and the like. Although up to 50% hydrogen-substituted compounds can be used, perhalo compounds are preferred. Most preferred are perfluorinated compounds.

Although any fluorochemical liquid i.e. a substance which is a liquid at about 20 degree.C at atmospheric pressure, can be used to prepare a fluorochemical emulsion of the present invention, for many purposes emulsions with longer extended stability are preferred. In order to obtain such emulsions, fluorochemical liquids with boiling points above 30.degree. C are preferred. Preferably the fluorochemical liquids have boiling points above 50 C., and most preferred are fluorochemical liquids with boiling points above about 90 C.

Useful perfluorocarbon emulsions are disclosed in U.S. Patent Nos. 4,927,623, 5,077,036, 5,114,703, 5,171,755, 5,304,325, 5,350,571, 5,393,524, and 5,403,575 and include those in which the perfluorocarbon compound is perfluorodecalin, perfluorooctane, perfluorodichlorooctane, perfluoro-n-octyl bromide, perfluoroheptane, perfluorodecane, perfluorocyclohexane, perfluoromorpholine, perfluorotripropylamine, perfluortributylamine, perfluorodimethylcyclohexane, perfluorotrimethylcyclohexane, perfluorodicyclohexyl ether, perfluoro-n-butyltetrahydrofuran, and compounds that are structurally similar to these compounds and are partially or fully halogenated (including at least some fluorine substituents) or partially or fully perfluorinated including perfluoroalkylated ether, polyether or crown ether.

Emulsifying agents, for example surfactants, may be used to facilitate the formation of emulsions. Typically, aqueous phase surfactants have been used to facilitate the formation of emulsions of fluorochemical liquids. A variety of lipid surfactants may be incorporated into the lipid monolayer preferably natural or synthetic phospholipids, but also fatty acids, cholesterols, lysolipids, sphingomyelins, tocopherols, glucolipids, stearylamines, cardiolipins, a lipid with ether or ester linked fatty acids, polymerized lipids, and lipid conjugated polyethylene glycol. The preferred surfactants for use in the practice of the invention are

phospholipids and cholesterol. Other known surfactants such as Pluronic F-68, Hamposyl.TM. L30 (W.R. Grace Co., Nashua, N.H.), sodium dodecyl sulfate, Aerosol 413 (American Cyanamid Co., Wayne, N.J.), Aerosol 200 (American Cyanamid Co.), Lipoproteol.TM. LCO (Rhodia Inc., Mammoth, N.J.), Standapol.TM. SH 135 (Henkel Corp.,

5   Teaneck, N.J.), Fizul.TM. 10-127 (Finetex Inc., Elmwood Park, N.J.), and Cyclopol.TM. SBFA 30 (Cyclo Chemicals Corp., Miami, Fla.); amphoterics, such as those sold with the trade names: Deriphat.TM. 170 (Henkel Corp.), Lonzaine.TM. JS (Lonza, Inc.), Niranol.TM. C2N-SF (Miranol Chemical Co., Inc., Dayton, N.J.), Amphoterge.TM. W2 (Lonza, Inc.), and Amphoterge.TM. 2WAS (Lonza, Inc.); non-ionics, such as those sold with the trade names:

10   Pluronic.TM. F-68 (BASF Wyandotte, Wyandotte, Mich.), Pluronic.TM. F-127 (BASF Wyandotte), Brij.TM. 35 (ICI Americas; Wilmington, Del.), Triton.TM. X-100 (Rohm and Haas Co., Philadelphia, Pa.), Brij.TM. 52 (ICI Americas), Span.TM. 20 (ICI Americas), Generol.TM. 122 ES (Henkel Corp.), Triton.TM. N-42 (Rohm and Haas Co.,), Triton.TM. N-101 (Rohm and Haas Co.,), Triton.TM. X-405 (Rohm and tlaas Co.,), Tween.TM. 80 (ICI

15   Americas), Tween.TM. 85 (ICI Americas), and Brij.TM. 56 (ICI Americas) and the like may be used. These surfactants' are used alone or in combination in amounts of 0.10 to 5.0% by weight to assist in stabilizing the emulsions.

          Fluorinated surfactants which are soluble in the fluorochemical liquid to be emulsified can also be used. Suitable fluorochemical surfactants include perfluorinated alkanoic acids

20   such as perfluorohexanoic and perfluorooctanoic acids and amidoamine derivatives. These surfactants are generally used in amounts of 0.01 to 5.0% by weight, and preferably in amounts of 0.1 to 1.0%. Other suitable fluorochemical surfactants include perfluorinated alcohol phosphate esters and their salts; perfluorinated sulfonamide alcohol phosphate esters and their salts; perfluorinated alkyl sulfonamide alkylene quaternary ammonium salts; N,N-

25   (carboxyl-substituted lower alkyl) perfluorinated alkyl sulfonamides; and mixtures thereof. As used herein, the term "perfluorinated" means that the surfactant contains at least one perfluorinated alkyl group.

          Suitable perfluorinated alcohol phosphate esters include the free acids of the diethanolamine salts of mono- and bis(1H,1H,2H,2H-perfluoroalkyl)phosphates. The

30   phosphate salts, available under the tradename "Zonyl RP" (E.I. Dupont de Nemours and Co., Wilmington, Del.), are converted to the corresponding free acids by known methods. Suitable perfluorinated sulfonamide alcohol phosphate esters are described in U.S. Pat. No. 3,094,547. Suitable perfluorinated sulfonamide alcohol phosphate esters and salts of these include perfluoro-n-octyl-N-ethylsulfonamidoethyl phosphate, bis(perfluoro-n-octyl-N-

ethylsulfonamidoethyl) phosphate, the ammonium salt of bis(perfluoro-n-octyl-N-
ethylsulfonamidoethyl)phosphate,bis(perfluorodecyl-N-ethylsulfonamidoethyl)-phosphate
and bis(perfluorohexyl-N ethylsulfonamidoethyl)-phosphate. The preferred formulations use
phosphatidylcholine, derivatized-phosphatidylethanolamine and cholesterol as the aqueous

5   surfactant.

        Lipid encapsulated emulsions can be formulated with cationic lipids in the surfactant
layer that facilitate the adhesion of nucleic acid material to particle surfaces. Cationic lipids
may include but are not limited to DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-
trimethylammonium chloride; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane;

10   DOTB,1,2-dioleoyl-3-(4'-trimethyl-ammonio)butanoyl-sn-glycerol, 1,2-diacyl-3-
trimethylammonium-propane; 1,2-diacyl-3-dimethylammonium-propane; 1,2-diacyl-sn-
glycerol-3-ethyl phosphocholine; and 3β-[N', N'-dimethylaminoethane)-
carbamol]cholesterol-HCl may be used. In general the molar ratio of cationic lipid to non-
cationic lipid in the lipid surfactant monolayer may be, for example, 1:1000 to 2:1,

15   preferably, between 2:1 to 1:10, more preferably in the range between 1:1 to 1:2.5 and most
preferably 1:1 (ratio of mole amount cationic lipid to mole amount non-cationic lipid, e.g.,
DPPC). A wide variety of lipids may comprise the non-cationic lipid component of the
emulsion surfactant, particularly dipalmitoylphosphatidylcholine, dipalmitoylphosphatidyl-
ethanolamine, 1,2-diacyl-sn-glycerol-3-phosphoethanolamine, cholesterol or

20   dioleoylphosphatidylethanolamine in addition to those previously described. In lieu of
cationic lipids as described above, lipids bearing cationic polymers such as polylysine or
polyarginine may also be included in the lipid surfactant and afford binding of a negatively
charged therapeutic, such as genetic material or analogues there of, to the outside of the
emulsion particles.

25   Release of nucleic acid constructs from cationic lipid emulsions can be induced by the
incorporation of anionic lipids from adjacent lipid monolayers and faciliated by the prolonged
contact and interactions afforded by ligand-targeted emulsions. A similar phenomena has
been reported when nucleic acid linked cationic liposomes become intracellular. These
anionic lipids form a charged ion-pair with the cationic lipids leading to the displacement of

30   oligonucleotide into the surrounding medium. Delivery of genetic material adsorbed onto the
surface of cationic emulsions can be enhanced by ligand-targeted emulsions and contact
facilitated delivery. Increased interaction and exchange of lipids from the target cell surface
to the outer membrane of the therapeutic particle will facilitate the transfer and release of
oligonucleotide.

In accordance with the present invention, the site-specific targeting ligand may be directly or indirectly conjugated to the surface of the emulsion particles. Direct conjugation of ligand to the emulsion particles refers to the preparation of a ligand-particle complex before administration to a patient wherein the ligand is either adsorbed through ionic,

5   electrostatic, hydrophobic or other noncovalent means to the particle surface (e.g. acylated-antibody), or chemically linked to the surface through covalent bonds to a component of the lipid surface such as a"primer material" (e.g. thio-ether or ester bond), or intrinsically incorporated into the lipid surfactant membrane as a component of the membrane (e.g. a lipid derivatized to a peptidomimetic agent).

10   Indirect conjugation refers to the use of avidin biotin wherein the complex is formed *in vivo* in two or more steps. For example, by administering the biotinylated antibody first, followed by avidin, and followed by the biotinylated emulsion particle. Any other sequential multistep chemical linking system that could be used *in vivo* is envisioned to produce the same end result, i.e. the close and specific apposition of the emulsion particle to a targeted

15   cell or tissue surface.

The emulsions of the present invention may be prepared by various techniques. One method is sonication of a mixture of oil or fluorochemical liquid and an aqueous solution containing a suitable "primer" material and/or specific binding species. Generally, these mixtures include a surfactant. Cooling the mixture being emulsified, minimizing the

20   concentration of surfactant, and buffering with a saline buffer will typically maximize both retention of specific binding properties and the coupling capacity of the primer material. These techniques provide excellent emulsions with high activity per unit of absorbed primer material or specific binding species.

When high concentrations of a primer material or specific binding species coated on

25   lipid emulsions, the mixture should be heated during sonication and have a relatively low ionic strength and moderate to low pH. Too low an ionic strength, too low a pH or too much heat may cause some degradation or loss of all of the useful binding properties of the specific binding species or the coupling capacity of the "primer" material. Careful control and variation of the emulsification conditions can optimize the properties of the " primer"

30   material or the specific binding species while obtaining high concentrations of coating.

Carbohydrate-bearing lipids may be employed for in vivo targeting, as described in U.S. Pat. No. 4,310,505, the disclosures of which are hereby incorporated herein by reference, in their entirety.

        While preparation of emulsions by sonication has been acceptable, some degree of
variability in particle size distribution may be observed. An alternative method of making the
emulsions involves directing high pressure streams of mixtures containing the aqueous
solution, a "primer" material or the specific binding species, the oil or fluorocarbon liquid
5   and a surfactant (if any) so that they impact one another to produce emulsions of narrow
particle size and distribution. The Microfluidizer™ apparatus (Microfluidics, Newton,
Mass.) can be used to make the preferred emulsions. The apparatus is also useful to post-
process emulsions made by sonication or other conventional methods. Feeding a stream of
emulsion droplets through the Microfluidizer™ apparatus yields formulations of small size
10   and narrow particle size distribution.
        Emulsifying and/or solubilizing agents may also be used in conjunction with
emulsions. Such agents include, but are not limited to, acacia, cholesterol, diethanolamine,
glyceryl monostearate, lanolin alcohols, lecithin, mono- and di-glycerides, mono-
ethanolamine, oleic acid, oleyl alcohol, poloxamer, peanut oil, palmitic acid, polyoxyethylene
15   50 stearate, polyoxyl 35 castor oil, polyoxyl 10 oleyl ether, polyoxyl 20 cetostearyl ether,
polyoxyl 40 stearate, polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 80,
propylene glycol diacetate, propylene glycol monostearate, sodium lauryl sulfate, sodium
stearate, sorbitan mono-laurate, sorbitan mono-oleate, sorbitan mono-palmitate, sorbitan
monostearate, stearic acid, trolamine, and emulsifying wax. All lipids with perfluoro fatty
20   acids as a component of the lipid in lieu of the saturated or unsaturated hydrocarbon fatty
acids found in lipids of plant or animal origin may be used. Suspending and/or viscosity-
increasing agents that may be used with emulsions include, but are not limited to, acacia,
agar, alginic acid, aluminum mono-stearate, bentonite, magma, carbomer 934P,
carboxymethylcellulose, calcium and sodium and sodium 12, carrageenan, cellulose, dextrin,
25   gelatin, guar gum, hydroxyethyl cellulose, hydroxypropyl methylcellulose, magnesium
aluminum silicate, methylcellulose, pectin, polyethylene oxide, polyvinyl alcohol, povidone,
propylene glycol alginate, silicon dioxide, sodium alginate, tragacanth, and xanthum gum.
        Useful emulsions may have a wide range of nominal particle diameters, e.g., from as
small as 0.01 microns to as large as 10 microns, preferably 0.1 to 0.5 micron diameter. The
30   emulsion particle sizes can be controlled and varied by modifications of the emulsification
techniques and the chemical components.
        The specific binding species (i.e. targeting ligand) may be immobilized on the
encapsulating lipid monolayer by direct adsorption to the oil/aqueous interface or using a
"primer material". A "primer material" is any surfactant compatible compound incorporated

in the particle to chemically couple with or adsorb a specific binding or targeting species i.e.
any constituent or derivatized constituent incorporated into the emulsion lipid surfactant layer
that could be chemically utilized to form a covalent bond between the particle and targeting
ligand or a component of the targeting ligand (if it has subunits). The preferred result is

5   achieved by forming an emulsion with an aqueous continuous phase and a biologically active
ligand adsorbed or conjugated to the "primer material" at the interface of the continuous and
discontinuous phases. Naturally occurring or synthetic polymers with amine, carboxyl,
mercapto, or other functional groups capable of specific reaction with coupling agents and
highly charged polymers may be utilized in the coupling process. The specific binding

10   species (e.g. antibody) may be immobilized on the emulsion particle surface by direct
adsorption or by chemical coupling. Examples of specific binding species which can be
immobilized by direct adsorption include small peptides, peptidomimetics, or polysaccharide-
based agents. To make such an emulsion the specific binding species may be suspended or
dissolved in the aqueous phase prior to formation of the emulsion. Alternatively, the specific

15   binding species may be added after formation of the emulsion and incubated with gentle
agitation at room temperature (25° C) in a pH 7.0 buffer (typically phosphate buffered saline)
for 1.2 to 18 hours.

Where the specific binding species is to be coupled to a "primer material",
conventional coupling techniques may be used. The specific binding species may be

20   covalently bonded to "primer material" with coupling agents using methods which are
known in the art. One type of coupling agent uses a carbodiimide such as 1-ethyl-3-(3-N,N
dimethylaminopropyl)carbodiimide hydrochloride or 1-cyclohexyl-3-(2-morpholinoethyl)
carbodiimide methyl-p-toluenesulfonate. The priimer material may be
phosphatidylethanolamine, N-caproylamine phosphatidylethanolamine, N-dodecanylamine

25   phosphatidylethanolamine, phosphotidylthioethanol, 1,2-diacyl-sn-glycerol-3-
phosphoethanolamine-N-[4-p-maleimidephenyl)-butyramide, N-succinyl-
phosphatidylethanolamine, N-glutaryl-phosphatidylethanolamine, N-dodecanyl-
phosphatidylethanolamine, N-biotinyl-phosphatidylethanolamine, N-biotinylcaproyl-
phosphatidylethanolamine, and phosphatidylethylene glycol. Other suitable coupling agents

30   include aldehyde coupling agents having either ethylenic unsaturation such as acrolein,
methacrolein, or 2-butenal, or having a plurality of aldehyde groups such as glutaraldehyde,
propanedial or butanedial. Other coupling agents include 2-iminothiolane hydrochloride,
bifunctional N-hydroxysuccinimide esters such as disuccinimidyl subsrate, disuccinimidyl
tartrate, bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, disuccinimidyl propionate,

ethylene glycolbis(succinimidyl succinate); heterobifunctional reagents such as N-(5-azido-2-
nitrobenzoyloxy)succinimide, p-azidophenylbromide, p-azidophenylglyoxal,4-fluoro-3-
nitrophenylazide, N-hydroxysuccinimidyl-4-azidobenzoate, m-maleimidobenzoyl N-
hydroxysuccinimide ester, methyl-4-azidophenylglyoxal, 4-fluoro-3-nitrophenyl azide, N-

5   hydroxysuccinimidyl-4-azidobenzoate hydrochloride, p-nitrophenyl 2-diazo-3,3,3-
trifluoropropionate, N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate, succinimidyl
4-(N-maleimidomethyl)cyclohexane-1-carboxylate, succinimidyl 4-(p-
maleimidophenyl)butyrate, N-succinimidyl(4-azidophenyldithio)propionate, N-succinimidyl
3-(2-pyridyldithio)propionate, N-(4-azidophenylthio)phthalamide; homobifunctional reagents

10   such as 1,5-difluoro-2,4-dinitrobenzene, 4,4'-difluoro-3,3'-dinitrodiphenylsulfone, 4,4'-
diisothiocyano-2,2'-disulfonic acid stilbene, p-phenylenediisothiocyanate, carbonylbis(L-
methionine p-nitrophenyl ester), 4,4'-dithiobisphenylazide, erythritolbiscarbonate and
bifunctional imidoesters such as dimethyl adipimidate hydrochloride, dimethyl suberimidate,
dimethyl 3,3'-dithiobispropionimidate hydrochloride and the like. Covalent bonding of a

15   specific binding species to the "primer material" can be carried out with the above reagents
by conventional, well-known reactions, for example, in the aqueous solutions at a neutral pH,
at temperatures of less than 25° C for 1 hour to overnight.

       Targeted therapeutic emulsions may incorporate bioactive agents (e.g drugs, genetic
materials, radioactive isotopes, or combinations thereof) in their native form or derivatized

20   with hydrophobic or charged moieties to enhance incorporation or adsorption to the ligand
targeted particle. Such therapeutics may include, but are not limited to antineoplastic agents,
such as platinum compounds (e.g., spiroplatin, cisplatin, and carboplatin), methotrexate,
fluorouracil,adriamycin, mitomycin, ansamitocin, bleomycin, cytosine arabinoside,
arabinosyl adenine, mercaptopolylysine, vincristine, busulfan, chlorambucil, melphalan (e.g.,

25   PAM, L-PAM or phenylalanine mustard), mercaptopurine, mitotane, procarbazine
hydrochloride dactinomycin (actinomycin D), daunorubicin hydrochloride, doxorubicin
hydrochloride, paclitaxel and other taxenes, rapamycin, manumycin A, TNP-470, plicamycin
(mithramycin), aminoglutethimide,estramustine phosphate sodium, flutamide, leuprolide
acetate, megestrol acetate, tamoxifen citrate, testolactone, trilostane, amsacrine (m-AMSA),

30   asparaginase (L-asparaginase) Erwina asparaginase, interferon .alpha.-2a, interferon .alpha.-
2b, teniposide (VM-26), vinblastine sulfate (VLB), vincristine sulfate, bleomycin sulfate,
hydroxyurea,procarbazine, and dacarbazine; mitotic inhibitors such as etoposide, colchicine,
and the vinca alkaloids, radiopharmaceuticals such as radioactive iodine and phosphorus
products; hormones such as androgens, progestins, estrogens and antiestrogens; anti-

helmintics, antimalarials, and antituberculosis drugs; biologicals such as immune serums, antitoxins and antivenoms; rabies prophylaxis products; bacterial vaccines; viral vaccines; aminoglycosides; respiratory products such as xanthine derivatives theophylline and aminophylline; thyroid agents such as iodine products and anti-thyroid agents; cardiovascular

5   products including chelating agents and mercurial diuretics and cardiac glycosides; glucagon; blood products such as parenteral iron, hemin, hematoporphyrins and their derivatives; biological response modifiers such as muramyldipeptide, muramyltripeptide, microbial cell wall components, lymphokines (e.g., bacterial endotoxin such as lipopolysaccharide, macrophage activation factor), sub-units of bacteria (such as Mycobacteria, Corynebacteria),

10   the synthetic dipeptide N-acetyl-muramyl-L-alanyl-D-isoglutamine; anti-fungal agents such as ketoconazole, nystatin, griseofulvin, flucytosine (5-fc), miconazole, amphotericin B, ricin, cyclosporins, and .beta.-lactam antibiotics (e.g., sulfazecin); hormones such as growth hormone, melanocyte stimulating hormone, estradiol, beclomethasone dipropionate, betamethasone, betamethasone acetate and betamethasone sodium phosphate, vetamethasone

15   disodium phosphate, vetamethasone sodium phosphate, cortisone acetate, dexamethasone, dexamethasone acetate, dexamethasone sodium phosphate, flunisolide, hydrocortisone, hydrocortisone acetate, hydrocortisone cypionate, hydrocortisone sodium phosphate, hydrocortisone sodium succinate, methylprednisolone, methylprednisolone acetate, methylprednisolone sodium succinate, paramethasone acetate, prednisolone, prednisolone

20   acetate, prednisolone sodium phosphate, prednisolone tebutate, prednisone, triamcinolone, triamcinolone acetonide, triamcinolone diacetate, triamcinolone hexacetonide, fludrocortisone acetate, oxytocin, vassopressin, and their derivatives; vitamins such as cyanocobalamin neinoic acid, retinoids and derivatives such as retinol palmitate, and .alpha.- tocopherol; peptides, such as manganese super oxide dismutase; enzymes such as alkaline

25   phosphatase; anti-allergic agents such as amelexanox; anti-coagulation agents such as phenprocoumon and heparin; circulatory drugs such as propranolol; metabolic potentiators such as glutathione; antituberculars such as para-aminosalicylic acid, isoniazid, capreomycin sulfate cycloserine, ethambutol hydrochloride ethionamide, pyrazinamide, rifampin, and streptomycin sulfate; antivirals such as acyclovir, amantadine azidothymidine (AZT, DDI,

30   Foscarnet, or Zidovudine), ribavirin and vidarabine monohydrate (adenine arabinoside, ara- A); antianginals such as diltiazem, nifedipine, verapamil, erythritol tetranitrate, isosorbide dinitrate, nitroglycerin (glyceryl trinitrate) and pentaerythritol tetranitrate; anticoagulants such as phenprocoumon, heparin; antibiotics such as dapsone, chloramphenicol, neomycin, cefaclor, cefadroxil, cephalexin, cephradine erythromycin, clindamycin, lincomycin,

amoxicillin, ampicillin, bacampicillin, carbenicillin, dicloxacillin, cyclacillin, picloxacillin, hetacillin, methicillin, nafcillin, oxacillin, penicillin including penicillin G and penicillin V, ticarcillin rifampin and tetracycline; antiinflammatories such as diflunisal, ibuprofen, indomethacin, meclofenamate, mefenamic acid, naproxen, oxyphenbutazone,

5   phenylbutazone, piroxicam, sulindac, tolmetin, aspirin and salicylates; antiprotozoans such as chloroquine,hydroxychloroquine, metronidazole, quinine and meglumine antimonate; antirheumatics such as penicillamine; narcotics such as paregoric;opiates such as codeine, heroin, methadone, morphine and opium; cardiac glycosides such as deslanoside, digitoxin, digoxin, digitalin and digitalis; neuromuscular blockers such as atracurium mesylate,

10   gallamine triethiodide, hexafluorenium bromide, metocurine iodide, pancuronium bromide, succinylcholine chloride (suxamethonium chloride), tubocurarine chloride and vecuronium bromide; sedatives (hypnotics) such as amobarbital, amobarbital sodium, aprobarbital, butabarbital sodium, chloral hydrate, ethchlorvynol, ethinamate, flurazepam hydrochloride, glutethimide, methotrimeprazine hydrochloride, methyprylon, midazolam hydrochloride,

15   paraldehyde, pentobarbital, pentobarbital sodium, phenobarbital sodium, secobarbital sodium, talbutal, temazepam and triazolam; local anesthetics such as bupivacaine hydrochloride, chloroprocaine hydrochloride, etidocaine hydrochloride, lidocaine hydrochloride, mepivacaine hydrochloride, procaine hydrochloride and tetracaine hydrochloride; general anesthetics such as droperidol, etomidate, fentanyl citrate with droperidol, ketamine

20   hydrochloride, methohexital sodium and thiopental sodium; and radioactive particles or ions such as strontium, iodide rhenium and yttrium.

         In addition, the ligand itself, such as an antibody, peptide fragment, or a mimetic of a biologically active ligand may contribute to the inherent therapeutic effects, either as an antagonistic or agonistic, when bound to specific epitopes. As an example, antibody against

25     $\alpha\nu\beta3$ integrin on neovascular endothelial cells has been shown to transiently inhibit growth and metastasis of solid tumors. The efficacy of therapeutic emulsion particles directed to the $\alpha\nu\beta3$ integrin may result from the improved antagonistic action of the targeting ligand in addition to the effect of the therapeutic agents incorporated and delivered by particle itself. Thus, in another embodiment of the invention, the targeting ligand and bioactive agent may

30   be constituted by a single component which functions both to target the ligand and to provide the bioactive agent to the desired site. As in the other embodiments previously described, when the targeting ligand and bioactive agent are constituted by a single component, such component must still be on or in the surface of the outer monolayer of the lipid encapsulated oil in water emulsion.

The following non-limiting examples illustrate the practice of the invention and the use of the lipid encapsulated oil in water emulsions with bioactive agents which are difficult to administer. By way of background for the examples set forth below, many potent pharmaceutical agents are difficult to administer systemically to patients in a safe and

5   efficacious manner either because of secondary complications due to toxicity of the parent drug or the formulation excipients. Many of these drugs are inherently hydrophobic, such as paclitaxel and analogues thereof. Other compounds are water soluble with lipophilic regions in the parent drug (e.g. doxorubicin) or are amenable to the addition of lipophilic moieties. In this disclosure, we reveal the enhanced effectiveness of such drugs incorporated into or on the

10   surfactant surface of emulsions, such as perfluorocarbon emulsions, targeted specifically to tissues, organs or cells through contact facilitated delivery.

Doxorubicin, is a antineoplastic agent that is incorporated into the treatment regimens of a wide variety of human tumors. Daunorubicin and its 14-hydroxy derivative, doxorubicin, are anthracycline antibiotics produced by the fungus streptomyces peucetius. Doxorubicin is

15   highly water soluble and structurally consists of an aglycon, adriamycinone, combined with an aminosugar, daunosamine Doxorubicin damages DNA by intercalation of the anthracycline portion, metal ion chelation, or by generation of free radicals. Doxorubicin has also been shown to inhibit DNA topoisomerase II which is critical to DNA function. Cytotoxic activity is cell cycle phase-nonspecific. The therapeutic activity of doxorubicin is

20   well validated, and equally well known are its adverse effects, including hair loss, mouth sores, nausea, vomiting, lowered blood counts (WBCs, RBCs and platelets), cardiotoxicity and skin damage secondary to extravasation during intravenous infusion. The anthracycline aspect of the molecule promotes the interaction of doxorubicin with acyl regions of lipid monolayers and bilayers, moderately with neutral layers and strongly with positive or

25   negatively charged membranes hydrophobic and electrostatic interactions. Doxorubicin has been encapsulated into liposomes with demonstrable improvements in organ distribution and effectiveness of the drug as well as a decrease of its cardiotoxicity versus free doxorubicin. The overall benefits of a variety of liposomal doxorubicin versus free drug independent of vesicle composition or formulation process are to decrease systemic toxicity symptoms

30   associated with high peak concentrations of drug and to prolong the pharmacological half-life of the compound.

In addition to liposomal Doxil, doxorubicin and its conjourners have been incorporated into a variety of emulsions and polymeric constructs with some success. Like the liposomal formulations, these formulations strive to administer high payloads of drug

with diminished peak concentrations and prolonged systemic half-life. For example, doxorubicin has been prepared as micelles composed of poly(ethylene glycol)-poly(beta-benzyl-L-aspartate) block copolymer (PEG-PBLA) by an o/w emulsion method with a substantial drug loading level (15 to 20 w/w%). Doxorubicin was chemically conjugated to a

5   terminal end group of poly(D,L-lactic-co-glycolic acid) [PLGA] and the doxorubicin-PLGA conjugate was formulated into nanoparticles to sustain the release of doxorubicin. Using sonication and a detergent, iodinated poppy seed oil (IPSO) has been mixed with aqueous solution epirubicin to yield a water-in-oil emulsion that is passed through a microporous glass membrane into saline water-in-oil-in-water emulsion (W/O/W) that consists of IPSO

10   microdroplets. A doxorubicin suspension has been prepared by emulsifying an aqueous solution directly into the lipid contrast medium, Lipiodol.

Another important antineoplastic agent is **paclitaxel** (i.e. taxol), a member of the class of compounds known as taxines, comes from the bark of the Pacific yew tree, Taxus brevifolia. For example, taxol has been used in treating ovarian, breast, non-small cell lung,

15   and head and neck carcinomas. One of the difficulties in administering taxol is that the drug is insoluble in water. The present state of the art in taxol formulation requires a 50:50 mixture of Cremophor-EL surfactant (polyoxyethylated castor oil) and ethanol in order to solubilize the drug. Unfortunately, this taxol formulation leads to a relatively high incidence of major hypersensitivity reactions (HSRs) upon intravenous administration. These HSRs have been

20   attributed to the unusually high concentration of Cremophor-EL required to solubilize the taxol.

There have been other attempts to provide a taxol formulation, the most successful of which has been incorporation of the drug into a liposomal formulation that must be freeze dried and reconstituted prior to use.

25   Attempts to formulate taxol in a stable lipid emulsion have been unsuccessful. Taxol is reported to be insoluble in lipid emulsions such as Intralipid.RTM., which contains soybean oil, or Liposyn.RTM., which contains a mixture of soybean and safflower oils. L. C. Collins-Gold et al., "Parenteral Emulsions for Drug Delivery", Advanced Drug Delivery Reviews, 5, pp. 189-208 (1990). Heating taxol in either soybean oil or safflower oil, even

30   upon sonication, does not result in the dissolution of appreciable amounts of taxol, and addition of taxol to a lipid emulsion during the homogenization step meets with equally disappointing results. Emulsions incorporating up to 15 mg/ml of taxol have been formulated with triacetin, L-.alpha.-lecithin, Polysorbate 80, Pluronic F-68, ethyloleate and glycerol.

However, these emulsions are highly toxic and unstable. B. Tarr et al., "A New Parenteral Emulsion for the Administration of Taxol", Pharmaceutical Research, 4, pp. 162-165 (1987).

Kaufman et al (U.S. patent no. 5,785,950) have recently overcome these issues and formulated an oil-in-water emulsion composed of taxine, an oil, water and a surfactant. In this

5   formulation, a taxine such as taxol is solubilized in the oil in an effective pharmaceutical amount for intravenous administration. The taxine and oil mixture forms a dispersed phase in the water. The oil may be any of a number of oils such as mineral, vegetable, animal, essential and synthetic oils, or mixtures thereof. Preferably, the oil is rich in triglycerides, such as safflower oil, soybean oil or mixtures thereof. Because taxol is more soluble in

10   safflower oil than soybean oil, safflower oil is most preferred. The surfactant used may be any of a number of surfactants, and usually is a phospholipid suchas lecithin. Typically, the taxine is present in an amount of about 0.1% to about 1% by weight of the emulsion, while the oil is present in an amount of from about 1% to about 40% and the surfactant is present in an amount of about 0.5% to about 5% by weight of the emulsion.

15   ***Example 1. Preparation of biotinylated doxorubicin emulsion.***

Targeting of doxorubicin therapeutic emulsions may be achieved with a three-step process for "pretargeting" a biotinylated antibody and subsequent binding of a biotinylated emulsion to a molecular epitope. The emulsion itself is produced by incorporating biotinylated phosphatidylethanolamine into the outer lipid monolayer of a perfluorocarbon

20   microemulsion. Briefly, the emulsion comprises perfluorooctylbromide (40% w/v, PFOB, 3M), a surfactant co-mixture (2.0%, w/v) and glycerin (1.7%, w/v). The surfactant co-mixture includes 64 mole% lecithin (Pharmacia Inc), 35 mole% cholesterol (Sigma Chemical Co.) and 1 mole% N-(6-(biotinoyl)amino) hexanoyl)-dipalmitoyl-L-alpha-phosphatidyl-ethanolamine, Pierce Inc.) which are dissolved in chloroform. Doxorubicin is suspended in

25   methanol (~25μg/20μl) and added in titrated amounts between 0.01 and 5.0 mol% of the 2% surfactant layer, preferably between 0.2 and 2.0mol%, The chloroform-lipid mixture is evaporated under reduced pressure, dried in a 50°C vacuum oven overnight and dispersed into water by sonication. The suspension is transferred into a blender cup (Dynamics Corporation of America) with perfluorooctylbromide and distilled, deionized water and

30   emulsified for 30 to 60 seconds. The emulsified mixture is transferred to a Microfluidics emulsifier (Microfluidics Co.) and continuously processed at 20,000 PSI for three minutes. The completed emulsion is vialed, blanketed with nitrogen and sealed with stopper crimp seal until use. A control emulsion can be prepared identically excluding doxorubicin from the

surfactant comixture. Particle sizes are determined in triplicate at 37°C with a laser light

scattering submicron particle size analyzer (Malvern Zetasizer 4, Malvern Instruments Ltd,

Southborough, MA)., which indicate tight and highly reproducible size distribution with

average diameters less than 400 nm. Unincorporated drug can be removed by dialysis or

5   ultrafiltration techniques.

Antibody of $F_{(ab)}$ fragment prepared as descrbed below is biotinylated using the EZ-

link™ Sulfo-NHS-LC-Biotinylation Kit. Briefly, 2 to 10 mg of protein in 1 ml of phosphate

buffered saline is combined with Sulfo-NHS-LC-Biotin in distilled or deionized water.to

afford a 12 to 20-fold molar excess of reagent to protein. Incubate solution in ce for 2 hours

10   or at room temperature for 30 minutes. Separate biotinylated protein from reagents using a

10 ml desalting column equilibrated and eluted with phosphate buffered saline. Collect

fractions of eluate and measure UV absorbance at 280 nm with a spectrophotometer. Store at

4°C until use.

Example 2. Preparation of antibody [F$_{(ab)}$]-conjugated doxorubicin emulsion.

15   The perfluorocarbon nanoparticle contrast agent is produced by incorporating 1,2-

dipalmitoyl-sn glycero-3-phosphoethanolamine-N-4 -(p-maleimidophenyl)butyramide (MPB-

PE) into the outer lipid monolayer of the emulsion to accommodate ligand conjugation. The

emulsion is comprised of perfluorooctylbromide (40% w/v), a surfactant co-mixture (2%

w/v), glycerin (1.2% w/v) and water (54.8% w/v). The surfactant co-mixture included

20   lecithin (67.9 mol%), cholesterol (30 mol%), dipalmitoyl-phosphatidylethanolamine (2

mol%) and MPB-PE (0.1 mol%) and is dissolved in chloroform. Doxorubicin is suspended in

methanol (~25μg / 20μl) and added in titrated amounts between 0.01 and 5.0 mol% of the 2%

surfactant layer, preferably between 0.2 and 2.0mol%. The chloroform-lipid mixture is

evaporated under reduced pressure, dried in a 50°C vacuum oven overnight and dispersed

25   into water by sonication. The suspension is transferred into a blender cup with

perfluorocarbon, safflower oil and distilled, deionized water and emulsified as above. A

control emulsion is prepared identically except a nonderivatized phosphatidylethanolamine

may be substituted into the surfactant co-mixture. The completed emulsion is vialed,

blanketed with nitrogen and sealed with stopper crimp seal until use. A control emulsion can

30   be prepared identically excluding doxorubicin from the surfactant comixture. Particle sizes

are determined in triplicate at 37°C with a laser light scattering submicron particle size

analyzer (Malvern Zetasizer 4, Malvern Instruments Ltd, Southborough, MA)., which

indicate tight and highly reproducible size distribution with average diameters less than 400 nm. Unincorporated drug is removed by dialysis.

F(ab)' fragments are generated and isolated using an immunopure F(ab)' preparation kit (Pierce, Rockford, IL). Briefly, IgG is dialyzed into 20mM phosphate/10mM EDTA buffer (pH 7.0), concentrated to 20 mg/ml and digested by immobilized papain. Solubilized F(ab)' is purified from Fc fragments and undigested IgG protein using a protein A column. F(ab)' fragments is purified from excess cysteine using a G25-150 column and deoxygenated phosphate buffer (pH 6.7). Fraction identity is confirmed by routine SDS-PAGE procedures. An analogous emulsion using an irrelevant IgG is used to prepare control ligands with random specificities.

F(ab)' fractions are pooled and combined with the MPB-PE derivatized emulsion (1-2 mg F(ab)'/ ml of emulsion). The mixture is adjusted to pH 6.7, sealed under nitrogen and allowed to react overnight at ambient temperatures with gentle, continuous mixing. The mixture is subsequently dialyzed with a 300,000 MWCO Spectra/Por DispoDialyzer (Laguna Hills, CA) against 10mM phosphate buffer (pH 7.2) to remove unconjugated F(ab)' fragments. The final emulsion is vialed under nitrogen and stored at 4° C until use. A nonspecific control emulsion may be prepared using the control, irrelevant IgG F(ab)' fragments in the above protocol.

Example 3. Effectiveness of therapeutic doxorubicin (DXR) nanoparticle emulsions targeted to tissue factor on porcine aortic smooth muscle cells.

Example 3 demonstrates the enhanced efficacy of ligand-targeted doxorubicin nanoparticle emulsions versus free doxorubicon to inhibit the proliferation of aortic smooth muscle cells *in vitro*. Pig aortic smooth muscle cells are seeded onto 12mm round glass coverslips in 24 well cluster plates at a density of 5 x10^4 cells per well (n=45; 9/treatment). The cells are grown in smooth muscle basal medium containing 5% FBS for 72 hours, then rinsed in media and incubated on a platform shaker at 37°C, 95%/5% O/CO2 with medium containing biotinylated anti-tissue factor antibody (25µg) for 1 hour. Excess antibody is rinsed from cultures 3x. Cells are next incubated with 25µg of avidin (Pierce, Rockford, IL) for 30 min then washed 3x to remove excess avidin. Finally, doxorubicin emulsion (25µl) incorporating 0, 0.2 or 2.0mol% drug within the phospholipid surfactant, is incubated with the cells for 30 minutes at 37°C. The cells were washed free of unbound emulsion or drug 3x and allowed to continue growing at 37°C, 95%/5% O/CO2. After 48 hours additional

growth, the cells were dispersed from the cover slips with trypsin and counted with a
hemacytometer (Fig. 1).

Tissue factor-targeted DXR nanoparticles had greater anti-proliferative effects than
the free doxorubicin and this differential effect was greater at the lower dosage of DXR.

5   DXR nanoparticles decreased cell proliferation in a dose responsive manner. The results
suggest that DXR formulated into the lipid surfactant was retained and slowly delivered from
the nanoparticles into the smooth muscle cells. The delivery of DXR into the pig aortic
smooth muscle cells is facilitated by the intimate, prolonged contact of the emulsion
nanoparticles with the cell membrane surface and exchange of lipids and drug between the

10   two lipid layers.

*Example 4. Effectiveness of therapeutic paclitaxel (Taxol) nanoparticle emulsions
targeted to tissue factor on porcine aortic smooth muscle cells.*

Example 4 demonstrates the efficacy of ligand-targeted paclitaxel nanoparticle
emulsions versus a targeted-control emulsion to inhibit the proliferation of aortic smooth

15   muscle cells *in vitro*. Since paclitaxel is highly insoluble in buffer, comparisons of
nanoparticle formulations with free drug were not possible. Pig aortic smooth muscle cells
were seeded onto 12mm round glass cover slips in 24 well cluster plates at a density of 5 x10^4
cells per well (n=18; 6/treatment). The cells were grown in smooth muscle basal medium
containing 5% FBS for 72 hours, then rinsed in media and incubated on a platform shaker at

20   37°C, 95%/5% O/CO_2 with medium containing biotinylated anti-tissue factor antibody
(25μg) for 1 hour. Excess antibody was rinsed from cultures 3x. Cells were next incubated
with 25μg of avidin (Pierce, Rockford, IL) for 30 min then washed 3x to remove excess
avidin. Finally, paclitaxel emulsions (25μl) at concentrations of 0, 0.2 and 2.0mol% within
the phospholipid surfactant, were incubated with the cell cultures for 30 minutes at 37C. The

25   cells were washed free of unbound emulsion (i.e. 3 rinses) and allowed to grow at 37°C,
95%/5% O/CO_2. After 48 hours, the cells were trypsinized and counted with a
hemacytometer (Fig. 2).

Tissue factor-targeted paclitaxel nanoparticles had marked, equivalent anti-
proliferative effects at both dosages of paclitaxel. Paclitaxel, although highly insoluble in

30   aqueous culture medium, was effectively delivered and inhibited the proliferation of aortic
smooth muscle cells. These results suggest that paclitaxel formulated into the lipid surfactant
was delivered from the nanoparticles directly into the smooth muscle cell membrane. The
delivery of paclitaxel to the pig aortic smooth muscle cells was facilitated by the intimate,

prolonged contact of the emulsion nanoparticles with the cell membrane surface and exchange of lipids and drug between the two lipid layers.

Example 5. Enhanced effectiveness of therapeutic doxorubicin or paclitaxel nanoparticle emulsions targeted to tissue factor on porcine aortic smooth muscle cells.

5   Example 5 demonstrates the enhanced effectiveness of ligand-targeted doxorubicin or paclitaxel nanoparticle emulsions versus non-targeted nanoparticle emulsion. This example illustrates the importance of ligand-targeting versus free emulsions. Pig aortic smooth muscle cells were seeded onto 12mm round glass cover slips in 24 well cluster plates at a density of 5 $\times 10^4$ cells per well (n=30; 3/treatment). The cells were grown in smooth muscle basal

10   medium containing 5% FBS for 72 hours, then rinsed in media and incubated on a platform shaker at 37°C, 95%/5% O/CO2 with medium. One half of the wells received biotinylated anti-tissue factor antibody (25μg) for 1 hour. Excess antibody and media was rinsed from all cultures 3x. One half of the cells were next incubated with 25μg of avidin (Pierce, Rockford, IL) for 30 min then washed 3x to remove excess avidin and media. Finally, paclitaxel,

15   doxorubicin or control (no drug) emulsions (25μl) at drug concentrations of 0, 0.2 and 2.0mol% within the phospholipid surfactant, were incubated with the cell cultures for 30 minutes at 37°C. The cells were washed free of unbound emulsion (i.e. 3 rinses) and allowed to grow at 37°C, 95%/5% O/CO2. After 48 hours, the cells were trypsinized and counted with a hemacytometer (Table 1).

20   Table 1.   The effect of targeted and free therapeutic nanoparticles on porcine aortic cell proliferation (cell counts x 10,000) *in vitro.*

Antibody	Control	Doxorubicin (DXR)		Paclitaxel(Taxol)	
		0.2mol%	2.0mol%	0.2mol%	2.0mol%
Free	23.3±1.8[a]	12.7±1.0[b]	7.9±0.3[c]	25.0±1.5[a]	23.0±1.5[a]
Targeted	27.0±1.2[a]	6.0±1.9[c]	1.9±0.7[d]	7.0±0.6[c]	5.2±0.4[c]

[abc] Means (± std error) with different superscripts are significantly different ($p<0.05$).

Tissue factor-targeted nanoparticles had greater anti-proliferative effects at both dosages of paclitaxel or doxorubicin than free therapeutic emulsion. The greatest benefit was measured for paclitaxel, the most hydrophobic compound. The control emulsions, free or

30   targeted did not inhibit cell proliferation. Free paclitaxel emulsion nanoparticles did not

affect cell growth regardless of dosage. Free doxorubicin emulsion particles did inhibit cell growth in a dose related fashion, but the effect was most pronounced when the particles were actively targeted to the cell surface with anti-tissue factor antibody, an epitope constitutively expressed by these cells. The delivery of paclitaxel or doxorubicin into the pig aortic smooth

5   muscle cells was facilitated by the intimate, prolonged contact of the emulsion nanoparticles with the cell membrane surface and the exchange of lipids and drug between the two lipid layers (see Fig. 3).

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above

10   description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.